PATENT
Docket No.: 201040/1020

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Alam et al.	Examiner: H. Schnizer
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For	:	HEME PROTEINS HEMAT-*HS* AND HEMAT-*BS* AND THEIR USE IN MEDICINE AND MICROSENSORS	

DECLARATION OF MAQSUDUL ALAM UNDER 37 CFR § 1.132

I, MAQSUDUL ALAM, pursuant to 37 C.F.R. § 1.132, declare that:

1. I received an M.S. in Microbiology from Moscow State University in 1979, a Ph.D. in Biochemistry from Moscow State University in 1982, and a Ph.D. in Biochemistry from Max-Planck Institute, Germany, in 1987.

2. I am currently employed as a Professor, Department of Microbiology, University of Hawaii at Manoa, Honolulu, Hawaii, and have served in that capacity since July 2001. Prior to my current position, I previously served as an Assistant Professor from August 1992 until July 1997 and as an Associate Professor from August 1997 until June 2001, also within the Department of Microbiology, University of Hawaii.

3. I am a co-inventor of the above-identified patent application.

4. I am presenting this declaration to demonstrate that (i) the complex as presently claimed in my above-referenced patent application contains a heme binding protein complexed with a heme molecule, and (ii) that the claimed complex is neither taught nor suggested by Zhang et al., "Signal Transduction in the Archaeon *Halobacterium salinarium* is Processed Through Three Subfamilies of 13 Soluble and Membrane-Bound Transducer Proteins," *Proc. Natl. Acad. Sci. USA* 93:4649-4654 (1996) ("Zhang").

5. When the HemAT-Hs and HemAT-Bs proteins taught in my application expressed in *E. coli* and purified (see Example 3), the media in which the host cells were grown contained a heme complex precursor, hemin, which allowed the host cells to produce the recombinant HemAT-Hs and HemAT-Bs proteins complexed with a heme molecule. The heme precursor increases the production of protoporphyrin IX, thus drastically enhancing the proper folding of the heme protein in host *E. coli*. The use of a heme synthesis precursor as a culture media additive for the production of a functional heme complexed protein was well known at the time my invention was made, as shown, for example, by the addition of hemin for the production of an active human hemoglobin tetramer (Hoffman et al., "Expression of Fully Functional tetrameric Human Hemoglobin in *Escherichia coli*," *Proc. Natl. Acad. Sci. USA* 87:8521-8525 (1990) (copy attached hereto as Exhibit 1)).

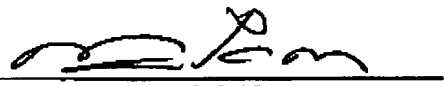
6. The use of such media for culturing heme complexed proteins was so routine that a skilled scientist, having read my patent application, would have understood from the teaching of the application that the recombinantly expressed proteins HemAT-*Hs* and HemAT-*Bs* had been cultured with heme precursors to form a protein-heme complex. This is evidenced by four characteristics of the complexes as disclosed in my application. First of all, a visible consequence of forming the protein-heme complex is that a solution containing the complex will be reddish brown in color. This is due to the iron molecule that lies at the core of the heme molecule. This feature is noted at page 22, lines 32-33, of the present application, which recites that the partially purified HemAT-*Hs* and HemAT-*Bs* proteins are described as a having a "brown red supernatant," indicating the presence of heme complexed with the protein. Secondly, the spectral properties of proteins containing a heme molecule were exhibited by the isolated complex of my patent application. It is well known that when a protein contains a heme molecule, that protein in its oxygenated form will typically display absorption band maxima that are similar to those of other heme binding proteins (i.e., those lacking a signal transducer domain) (*see* Example 4 at pages 24-25). The purified HemAT-*Hs* complexed with heme exhibited the following absorption band maxima: Soret band at 406 nm, α-band at 578 nm, and β-band at 538 nm (*see* Example 4 at page 24, lines 20-22, and Figure 4A). Thirdly, a pyridine hemochrome assay performed on the complexes confirmed that the "heme group of both HemAT-*Hs* and HemAT-*Bs* ...[is the] ... b-type (*see* Example 4 at page 24, line 32 to page 25, line 1). The fourth piece of evidence is the spectral characteristics of the complexes in response to sodium dithionite, which

deoxygenates an oxygen-bound heme molecule, and subsequent exposure of the deoxygenated complex to atmospheric oxygen, which causes reversion to the previously noted absorption pattern (*see* Example 4 at pg. 24, lines 24-29, and Figures 4B, 4D). From the foregoing, it is clear from the data provided in my application that the isolation of HemAT-*Hs* and HemAT-*Bs* proteins as taught in my patent application produced in each instance an isolated heme-binding protein complexed to a heme molecule.

7. I am a coauthor of Zhang and, therefore, I am highly familiar with the subject matter thereof. Zhang relates to the identification ofa putative signal transduction gene family from *H. salinarium*, and discloses the partial purification of several methyl-accepting taxis halobacterial proteins of that family. Although some physical properties of the HtB protein were disclosed in Zhang, my coauthors and I had yet to discover that HtB was a heme-binding protein and, therefore, could form a complex with heme. In fact, at the time of publishing Zhang, the function of HtB was not known (*see* Zhang, Figure 3 legend). Given that it was unknown that HtB was a heme-binding protein, heme complex precursors were never added to the culture media when growing the bacterium used for the *H. salinarium* signal transducer protein localization studies as reported Zhang. As a result, none of the partially purified *H. salinarium* signal transducer proteins disclosed in Zhang were complexed with a heme molecule (i.e., there is no report in Zhang of any brown red color in the cell fractions containing the soluble proteins such as HtB). From all of the above, it is clear that the HtB *H. salinarium* signal transducer protein disclosed in Zhang is not in the form of a complex as presently claimed in the above-identified patent application.

8. I hereby declare that all statements made herein of my own knowledge are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

JUNE 21, 2004
Date



Maqsudul Alam

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Biochemistry

Expression of fully functional tetrameric human hemoglobin in *Escherichia coli*

(synthetic gene/gene coexpression/blood substitute)

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Communicated by Irving M. Klotz, August 15, 1990

ABSTRACT **Synthetic genes encoding the human α- and β-globin polypeptides have been expressed from a single operon in *Escherichia coli*. The α- and β-globin polypeptides associate into soluble tetramers, incorporate heme, and accumulate to >5% of the total cellular protein. Purified recombinant hemoglobin has the correct stoichiometry of α- and β-globin chains and contains a full complement of heme. Each globin chain also contains an additional methionine as an extension to the amino terminus. The recombinant hemoglobin has a C_4 reversed-phase HPLC profile essentially identical to that of human hemoglobin A_0 and comigrates with hemoglobin A_0 on SDS/PAGE. The visible spectrum and oxygen affinity are similar to that of native human hemoglobin A_0. The recombinant protein shows a reduction in Bohr and phosphate effects, which may be attributed to the presence of methionine at the amino termini of the α and β chains. We have also expressed the α- and β-globin genes separately and found that the expression of the α-globin gene alone results in a marked decrease in the accumulation of α-globin in the cell. Separate expression of the β-globin gene results in high levels of insoluble β-globin. These observations suggest that the presence of α- and β-globin in the same cell stabilizes α-globin and aids the correct folding of β-globin. This system provides a simple method for expressing large quantities of recombinant hemoglobin and allows facile manipulation of the genes encoding hemoglobin to produce functionally altered forms of this protein.**

The prevalence of infectious agents in human blood products, such as red blood cells, has changed modern transfusion practice. This fact, and the need for an oxygen-carrying solution that can be administered quickly to patients requiring acute blood transfusion, has led to a continued interest in developing red-cell substitutes. The primary functional requirement of such substitutes is that they not only provide blood volume but also adequately deliver oxygen to the tissues. The use of cell-free solutions of hemoglobin (Hb) as a potential oxygen-carrying red-cell substitute has been investigated for decades (1). Purified Hb has seen shown to efficiently transport oxygen in animals without appreciable toxicity (2). The use of unmodified human Hb purified from red blood cells suffers from four limitations. (*i*) Hb purified from red blood cells loses a cofactor [2,3-bis(phospho)glycerate] resulting in an increase in the oxygen affinity. (*ii*) The dissociation of Hb tetramers into dimers leads to the rapid clearance of Hb from the circulatory system, resulting in a short circulating half-life and possibly renal toxicity (3). (*iii*) Hb solutions derived from red cells may be contaminated with infectious agents. While sterilization of red blood cell-derived Hb may be possible, it is often difficult to achieve without compromising the functionality of the protein. (*iv*) The source of Hb from outdated units of human red blood cells is susceptible to supply limitation. The first two problems have been addressed by chemical modifications of Hb that alter its oxygen delivery characteristics and circulating half-life (4). Alternatively, recombinant Hb could be modified by the techniques of protein engineering to modulate oxygen affinity and stabilize the protein from dissociation, thus avoiding problems attributable to chemical modification of Hb. In addition, production of Hb by fermentation could ensure a consistent supply of a protein free of blood-borne pathogens. For these reasons, we have developed a system that allows high-level production of human Hb in *Escherichia coli*.

Production of totally recombinant Hb presents a challenge in that it requires correct assembly of two nonidentical α and β subunits, followed by introduction of a reduced heme cofactor. A method for the production and engineering of semisynthetic human Hb in *E. coli* was described by Nagai *et al.* (5). This method involved the synthesis of β-globin fusion proteins with a cleavable linker sequence. The fusion protein was purified, cleaved to release the globin chain, and refolded *in vitro* in the presence of native α-globin and heme to gain a fully functional tetramer. We have developed an *E. coli* expression system that results in the expression of both globin chains. The α- and β-globin chains fold *in vivo* and incorporate endogenous heme, with recombinant Hb accumulating in the soluble cytoplasmic fraction of the cell.

MATERIALS AND METHODS

Construction of a Synthetic Hb Operon. Synthetic oligodeoxynucleotides were synthesized using a Biosearch model 8600 synthesizer. Codons were chosen based on the *E. coli* codon bias (6). In some cases the choice of codons was dictated by the need to include convenient restriction sites. Oligonucleotides were purified by preparative polyacrylamide gel electrophoresis in 20% acrylamide gels containing 7 M urea in 1× TBE (90 mM Tris borate, pH 8.3/2 mM Na_2EDTA). Regions of the gel containing the full-length oligonucleotide were excised and incubated in 0.1 M Tris·HCl, pH 7.8/0.5 M NaCl/5 mM Na_3EDTA at 60°C for 16 hr. Gel fragments were removed by centrifugation (14,000 × *g*, 5 min) and the solution containing the oligonucleotide was loaded onto a C_{18} Sep-Pak cartridge (Waters) equilibrated in water. The bound oligonucleotide was washed with 20 ml of water and eluted with 3 ml of 25 mM triethylammonium acetate, pH 7.3, in 50% methanol. The purified oligonucleotide was lyophilized, washed with 100% ethanol, and resuspended in water. Each globin gene was constructed from 14 separate oligonucleotides ranging in length from 50 to 85

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Abbreviation: IPTG, isopropyl β-D-thiogalactopyranoside.

*To whom reprint requests should be addressed.

nucleotides. Only the two oligonucleotides corresponding to the 5′ ends of each construct were not phosphorylated. Each gene was assembled, separately, in a single annealing and ligation reaction (7). The fragment corresponding to the α-globin gene was cloned into bacteriophage vector M13mp-19 (8) that had been digested with *Xma* I and *Pst* I. The β-globin fragment was cloned into *Pst* I- and *Hin*dIII-digested M13mp19. Nucleotide sequencing (9) of M13 transfectants was used to identify isolates containing genes with the correct sequence. The α- and β-globin genes were then cloned sequentially into the plasmid pKK223-3 (Pharmacia) as *Eco*RI–*Pst* I and *Pst* I–*Hin*dIII fragments, respectively. The resulting plasmid (pDLIII-13e, Fig. 1) contains the two globin genes cotranscribed from the P_{tac} promoter (10). The translational initiation site for the α-globin cistron is that described by Schoner *et al.* (11). The translational coupler used to link the α-globin and β-globin cistrons is shown in Fig. 1. In addition to pDLIII-13e, plasmids designed to express either α-globin or β-globin alone were also constructed from pKK223-3. These constructs (pDLII-86c and pJRIV-50a, respectively) use the same translational initiation site as in the respective genes in pDLIII-13e. *E. coli* JM109 was transformed (12) with the plasmids described above.

Purification of Hb. *E. coli* JM109(pDLIII-13e) was grown to 1.5–3 × 10^9 cells/ml at 30°C in TB medium (2.4% yeast extract/1.2% tryptone/0.4% glycerol/0.17 M KH_2PO_4/0.72 M K_2HPO_4) supplemented with hemin (40 μg/ml). Expression of globin was induced by the addition of 300 μM isopropyl β-D-thiogalactopyranoside (IPTG). At the time of induction the solution was brought to 1% glucose. Four hours after induction, cells were harvested by centrifugation

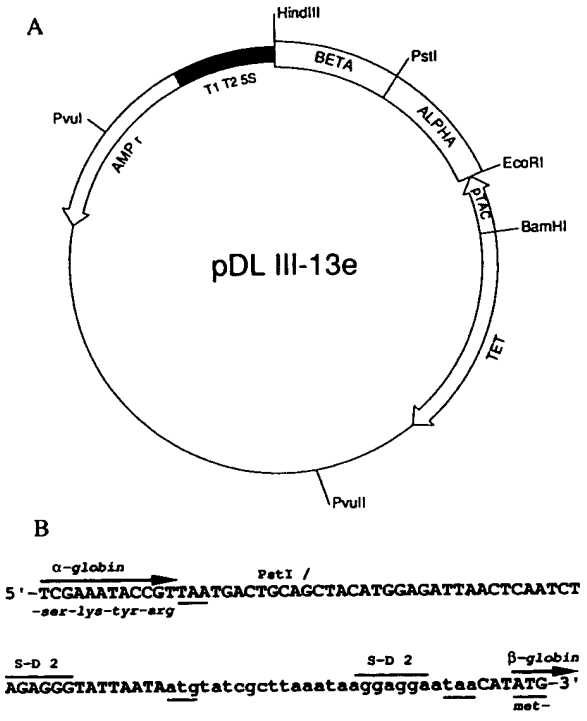

FIG. 1. (*A*) Recombinant Hb expression plasmid pDLIII-13e, which was derived from pKK223-3 (Pharmacia). (*B*) Nucleotide sequence at the junction of α- and β-globin genes. The 3′ end of the α-globin gene and 5′ end of the β-globin gene are shown with corresponding amino acid sequence. The translational initiation codon (ATG) and the translational stop codon (TAA) are underlined. The Shine–Dalgarno (S–D) ribosome binding site sequences are overlined, and the translational coupler sequence (11) is designated by lowercase letters.

(10,000 × *g*, 10 min, 4°C) and resuspended in 40 mM sodium phosphate (pH 7.0) (3 ml/g of cell paste). Aprotinin [200 kallikrein inhibitory units (KIU)/ml], DNase I (20 mg/ml), and lysozyme (2 mg/g of cell paste) were added, and the cells were broken by four passes through a Dynomill cell disrupter (Glen Mills, Maywood, NJ). The suspension was centrifuged (14,000 × *g*, 45 min, 4°C) and the supernatant, containing recombinant Hb, was bound to a dextran sulfate-Sephadex (Pharmacia) column (5 × 7 cm, equilibrated in 40 mM sodium phosphate, pH 7.0). After loading, the column was washed with 10 mM sodium phosphate (pH 7.0) containing aprotinin (100 KIU/ml). Hb was eluted with 20 mM Tris·HCl (pH 7.5) containing aprotinin (100 KIU/ml). The eluted Hb was concentrated with a Centricon-30 concentrator (Amicon) and loaded onto a 20-ml Mono Q FPLC column (Pharmacia). Hb was eluted with a 240-ml linear gradient of 0–0.16 M NaCl in 20 mM Tris·HCl (pH 8.0). The major Hb peak was eluted at 40 mM NaCl. Fractions containing Hb were concentrated and the buffer was exchanged by diafiltration (Centricon-30). Hb in 10 mM sodium phosphate (pH 7.0) was loaded onto a 20-ml Mono S FPLC column (Pharmacia) equilibrated in the same buffer. Hb was eluted with a 200-ml linear gradient from 10 mM sodium phosphate at pH 7.0 to 0.16 M NaCl/10 mM sodium phosphate at pH 9.0.

Human Hb A_0 was purified from red blood cells by modifications of existing procedures (13) and the methods described above.

Separation of α- and β-Globin Chains and Protein Sequencing. The α- and β-globin chains from recombinant Hb were separated by reverse-phase HPLC on a Vydac C_4 column (25 × 0.46 cm) with a gradient of 30–60% acetonitrile containing 0.1% trifluoroacetic acid. Protein sequence data were obtained from nonreduced, HPLC-purified samples by using an Applied Biosystems model 477A protein sequencer, followed by HPLC analysis of phenylthiocarbamoyl-derivatized amino acids (14).

Analytical Methods. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce) with crystallized human Hb or egg white lysozyme as standard. The concentration of Hb in solution was determined using published extinction coefficients (13). Heme content was estimated by integrating the peaks obtained from reverse-phase HPLC (see above) and comparison to known standards. Methemoglobin was determined by the method of Drabkin and Austin (15).

The method of Laemmli (16) was used for polyacrylamide gel electrophoresis of proteins. Electrophoresis of nucleic acids was as described by Maniatis *et al.* (12).

Oxygen Affinity Measurements. Oxygen equilibrium curves were measured in a Hemox analyzer (TCS Medical Products, Southampton, PA) at 25.0 ± 0.1°C in 50 mM Hepes/0.1 M NaCl. Oxygen equilibrium curves were measured by N_2 deoxygenation of oxyhemoglobin previously equilibrated with water-saturated O_2 or air. Both the absorbance and the oxygen pressure were sampled by a programmable-gain 12-bit analog-to-digital converter (Labmaster PGH; Scientific Solutions Solon, OH) under computer control. The oxygen equilibrium curve was subjected to a low-pass digital filter. P_{50} values (partial pressure of O_2 required for 50% saturation) and Hill coefficients (n_{max}) were calculated from the digitally filtered data by using software developed in our laboratory. The Hill coefficients were determined as the maximum slope of the function $d \log[y/(1-y)]/d \log p$ (17).

RESULTS

Coexpression of α- and β-Globin Genes in *E. coli*. To achieve coexpression of stoichiometric amounts of α- and β-globin, we designed a Hb operon. Synthetic α- and β-globin genes were assembled and cloned into the pKK223-3 expres-

sion vector to create pDLIII-13e (Fig. 1*A*). The operon, under the regulation of the P_{tac} promoter (10), consisted of two pairs of cistrons, one each for α- and β-globin. Cotranslational coupling sequences were used to increase ribosomal loading (Fig. 1*B*). Each globin gene contained separate ribosome binding and translational initiation and termination sites. The α- and β-globin genes were synthesized using an *E. coli* codon bias to enhance expression (18) and with restriction endonuclease sites regularly placed to aid in the construction of altered forms of Hb. Analogous expression vectors carrying only the α- and β-globin gene were constructed to allow comparison of coexpression with expression of the genes separately.

E. coli JM109 cultures transformed with these plasmids were grown to midlogarithmic phase and globin synthesis was induced by the addition of IPTG. Cells were harvested 4 hr after the addition of inducer and analyzed by SDS/PAGE (Fig. 2). Cells bearing pDLIII-13e accumulated two polypeptides, in approximately equal amounts, that comigrated with α- or β-globin. Cells bearing the β-globin expression vector produced, as expected, a polypeptide that comigrated with authentic β-globin. *E. coli* JM109 transformed with the α-globin expression vector, however, did not produce detectable quantities of α-globin as assayed by staining with Coomassie brilliant blue. We were able to detect trace quantities of α-globin when immunological staining procedures were used (data not shown).

Separation of *E. coli* lysates into soluble and insoluble fractions (Fig. 3) demonstrated that coexpressed α- and β-globin were found predominantly in the soluble fraction. In cells producing only β-globin, however, the protein was found primarily in the insoluble fraction (D.L.L., unpublished data). Based on the absence of pigmented material, it appears that heme was not incorporated into β-globin under these conditions. These results suggest that a synergistic action between α- and β-globin may affect correct folding of

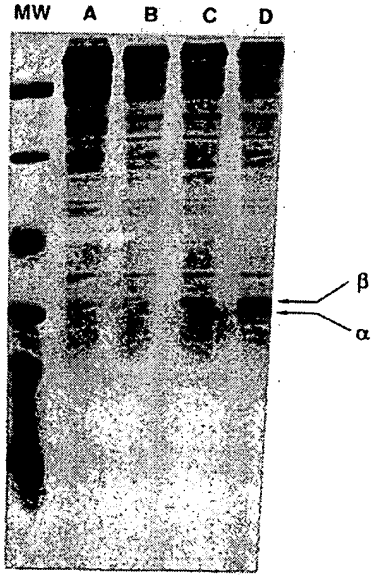

FIG. 2. Expression levels of α-globin and β-globin and coexpression of α- and β-globin. *E. coli* (10^8 cells) were solubilized in 3% SDS/5% 2-mercaptoethanol/5% glycerol/62.5 mM Tris·HCl, pH 6.8, at 95°C for 10 min. The cell solution was subjected SDS/15% PAGE and proteins were visualized by staining with Coomassie brilliant blue. Lane MW, molecular weight markers (from top to bottom, M_r 43,000, 29,000, 18,400, 14,300, 6200, and 3000); lane A, uninduced JM109(pDLIII-13e); lane B, induced JM109(pDLII-86c); lane C, induced JM109(pJRIV-50a); lane D, induced JM109(pDLIII-13e).

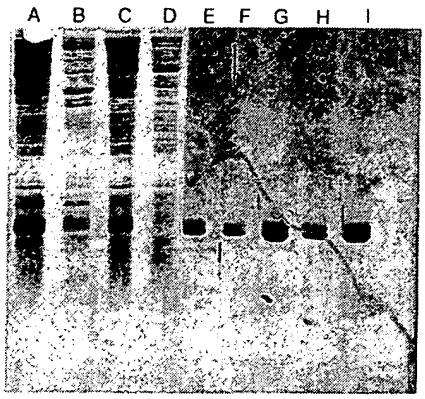

FIG. 3. Purification of recombinant Hb. Lanes: A, total cell lysate; B, insoluble material from centrifugation of total cell lysate; C, clarified lysate following centrifugation; D, flow-through from the dextran sulfate-Sephadex column; E–I, recombinant Hb fractions from the dextran sulfate column (lane E), Q-Sepharose column (lanes F and G), and S-Sepharose column (lanes H and I). Lanes A–D were loaded with 5–10 μg of protein. Lanes E–I were loaded with 2.5–5 μg of protein.

the two globin polypeptides in *E. coli*. When clarified lysates prepared from IPTG-induced cells containing pDLIII-13e were bubbled with carbon monoxide, the lysate developed a bright red color, suggesting that the two globin polypeptides were properly assembled and had incorporated heme. Immunological quantitation (Western blot) and densitometric scans of Coomassie blue-stained polyacrylamide gels indicated that coexpression of α- and β-globin resulted in accumulation to 5–10% of the total cell protein.

Characterization of Recombinant Hb. Recombinant Hb was purified from the soluble fraction of *E. coli* lysates by sequential chromatography on dextran sulfate, Mono Q, and Mono S resins. Chromatography on Mono Q of pooled recombinant Hb from dextran sulfate-Sephadex resolved three Hb species. Approximately 25% of the material was found in a peak that contained methemoglobin. Another 25% was found in a peak containing Hb with <4 mol of heme per mol of Hb as determined by C_4 HPLC. The major peak from this column, however, contained Hb with a full complement of heme with >90% reduced heme iron. Recombinant Hb eluted from the Mono Q was purified on Mono S to remove trace impurities. When saturated with oxygen, recombinant Hb had a visible spectrum identical to that of human Hb A_0, with absorbance maxima at 540 and 576 nm. Reverse-phase HPLC (Fig. 4) of recombinant Hb and Hb A_0 monitored at 280 nm demonstrated that the recombinant material contained the correct heme and globin stoichiometry and that the recombinant globins had chromatographic characteristics similar to those of the native proteins. Drabkin analysis (15) of purified recombinant Hb revealed <5% methemoglobin.

The α- and β-globin chains of recombinant Hb were separated by C_4 HPLC and the sequence of the first six amino acids of both chains was determined (data not shown). The sequence obtained from both α- and β-globin indicated that the methionine from the translational initiation codon was not efficiently removed. For α-globin the sequence was Met-Val-Leu-Ser-Pro-Ala. A secondary sequence was obtained for α-globin, beginning with valine, which suggested that the amino-terminal methionine was removed from about 10% of the α-globin protein. The sequence obtained from β-globin indicated that the translational initiator methionine was not removed to any detectable level, and corresponded to the expected amino-terminal sequence: Met-Val-His-Leu-Thr-Pro.

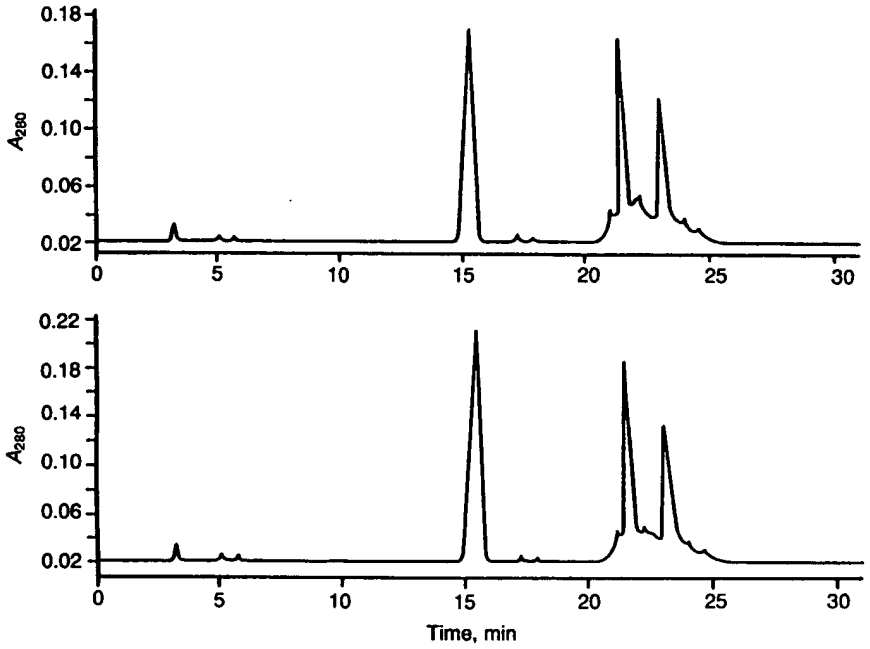

FIG. 4. C_4 reverse-phase chromatography of Hb A_0 (*Upper*) and recombinant Hb (*Lower*). The peaks at 15.5, 21.6, and 23.2 min correspond to heme, β-globin, and α-globin, respectively, monitored at 280 nm.

Oxygen Affinity of Recombinant Hb. Purified recombinant Hb was analyzed for oxygen affinity, cooperativity, Bohr effect, and response to organic phosphate. Measured oxygen binding properties (Fig. 5) of the recombinant protein were very similar to those of human Hb A_0. P_{50} (50 mM Hepes, pH 7.4/0.1 M NaCl; 25°C) of recombinant Hb was 3.3 torr, compared to 3.8 torr for Hb A_0; Hill coefficients (n_{max}) were 2.5 and 2.7, respectively (Table 1). Thus, the additional methionine residue found on α- or β-globin subunits does not significantly alter either the oxygen affinity or the allosteric behavior of this protein. Because the Hill value is a measure of Hb cooperativity, the apparent high cooperativity of recombinant Hb lends additional support to evidence that globin and heme have been properly assembled into the bacterially synthesized protein. The Bohr effect of recombinant Hb (Fig. 6 *Upper*) was reduced by ≈50%. A possible explanation is that the proton-mediated effect on oxygen affinity was partially disrupted by the additional amino-terminal residue on α- and/or β-globin. The effect of pH on the cooperativity of recombinant Hb was not, however, significantly affected by the additional residue (Fig. 6 *Lower*). Inositol hexakisphosphate binding lowered the affinity for oxygen, demonstrating that the phosphate binding site in the β cleft was functional (Table 1). The presence of the additional methionine on recombinant Hb, however, appeared to decrease the response to inositol hexaphosphate relative to Hb A_0.

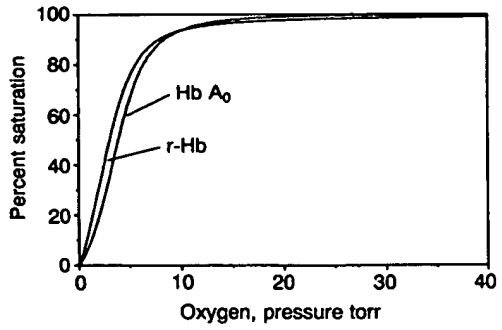

FIG. 5. Oxygen equilibrium curves comparing recombinant Hb (r-Hb) and Hb A_0 in 50 mM Hepes, pH 7.4/0.1 M NaCl at 25 ± 0.1°C. (1 torr = 133 Pa.)

DISCUSSION

In many respects, *E. coli* is an excellent choice of organism in which to attempt to express heteromultimeric proteins. The organization of most *E. coli* genes into operons suggests an obvious way to attempt production of proteins that consist of several different polypeptides—namely, the construction of synthetic operons in which the expression of the subunits is co-regulated. Several heteromultimeric proteins have been produced in *E. coli*, including the hexadecameric ribulose-bisphosphate carboxylase (19), immunoglobulin Fv fragments (20), and chimeric Fab fragments (21). Our Hb operon was designed to overcome difficulties in producing stoichiometric amounts of the two globin chains. This was apparently successful. An equivalent level of mRNA coding sequences alone is unlikely to provide the basis for this success. The plasmid designed for coexpression of α- and β-globin produces an mRNA molecule with potentially different secondary structure than mRNA coding only for α- or β-globin. These differences could result in variations in mRNA stability or translation efficiency. However, the striking difference in polypeptide levels seen when α- and β-globin genes were expressed alone suggests that posttranscriptional effects involving α/β-globin interactions may be critical to protein folding and stability. It is clear that coexpression of α- and β-globin influences the intracellular distribution of β-globin.

Table 1. Effects of inositol hexakisphosphate (IHP) on oxygen binding to recombinant Hb (r-Hb) and human Hb A_0

Protein	P_{50}, torr		n_{max}
	+ IHP	− IHP	
Hb A_0	15.6	3.8	2.7
r-Hb	10.2	3.3	2.5

P_{50} was measured at 25 ± 0.1°C in 50 mM Hepes, pH 7.4/0.1 M NaCl with or without 35 μM IHP as indicated.

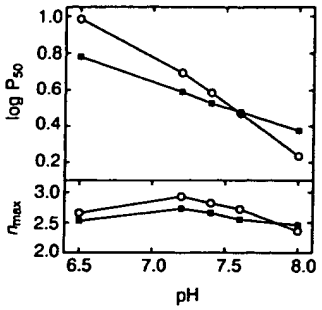

FIG. 6. Oxygen-binding properties of recombinant Hb (■) and human Hb A_0 (○). P_{50} values and Hill coefficients (n_{max}) were obtained in 50 mM Hepes buffer/0.1 M NaCl, 25 ± 0.1°C, at pH 6.5, 7.2, 7.4, 7.6, and 8.0.

In the absence of α-globin, β-globin is insoluble, most probably is not correctly folded, and does not appear to contain heme. Increased accumulation of α-globin when synthesized in the presence of β-globin may reflect the increased stability of correctly folded α-globin when incorporated into a tetramer. Yip *et al.* (22) have suggested that proper folding of globin chains is enhanced by the presence of both globin chains.

Like other heme-containing proteins expressed in *E. coli* (23, 24), recombinant Hb is capable of incorporating heme provided by the host cell. However, FPLC resolution of a recombinant Hb fraction that is deficient in heme suggests that endogenous heme biosynthesis may not be sufficient to supply the demand for heme completely in response to the induction of the globin genes.

Recombinant human Hb produced in *E. coli* is nearly identical to human Hb A_0 in terms of oxygen affinity, cooperativity, and the effect of physiologic modifiers on oxygen affinity. Recombinant Hb displays cooperative oxygen binding, with a Hill coefficient nearly equal to that of Hb A_0, indicative of a properly assembled and functional molecule. The major differences between Hb A_0 and recombinant Hb are in the response of the recombinant protein to physiologic modifiers such as hydrogen ion (Bohr effect) and organic phosphate. The Bohr effect is known to be partially mediated through the amino terminus of α-globin (25). Because the α chains retain the initiating methionine residue, a reduced Bohr effect is not surprising. Likewise, the response to phosphate is known to involve the β-chain amino-terminus. The human variant Hb Long Island (26), containing a histidine-to-proline change at β position 2, also exhibits a reduced phosphate response. Although the altered response might be due directly to the amino acid substitution, another consequence of this mutation is that the β-globin amino-terminal methionine is retained. The additional amino-terminal residue or its bulky side group might also partially obstruct the β cleft, preventing access by phosphate.

It is unclear why methionine is not more efficiently removed from the amino termini of recombinant Hb. The penultimate residue in both chains is valine, a residue that ordinarily does not interfere with processing by the prokaryotic methionyl aminopeptidase (27). It is possible that the failure to remove the methionine is due to obstruction of the processing enzyme by an early folding intermediate formed while the nascent polypeptides are still bound to the ribosome. This would have to occur early in translation, since methionyl aminopeptidase is thought to process the methionine cotranslationally, when the nascent chain is between 15 and 20 residues in length (28). Both chains have α-helical regions that occur within the first 20 amino acids that could obstruct processing by the enzyme. Deformylase, which removes the formyl group from the initiating methionine, does appear to have access to the growing chain, as neither α- nor β-globin appear to have modified amino termini.

The ability to produce functional recombinant Hb *de novo* in the system described in this paper facilitates the production of Hb mutants (S.J.H., D.L.L., and G.L.S., unpublished data). Specific amino acid substitutions incorporated into recombinant Hb modeling naturally occurring mutants (29) or rationally designed Hb variants (30) could be used to modulate oxygen affinity. Other specific mutations could be used to stabilize Hb to dissociation and oxidation. Such genetically engineered Hb could provide advantages over mammalian Hb for use in a red-cell substitute.

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